EGFR 37 KDA FRAGMENT AS CANCER MARKER

The present invention relates to a method of diagnosis of bladder cancer or prostate cancer and to a method of detecting recurrence of bladder or prostate cancer. More particularly the invention relates to an accessible marker.

Transitional cell carcinoma (TCC) of the bladder accounts for 1% of all cancers and is the fifth most common malignancy in people over the age of sixty in industrialised parts of the world (Russell et al., 1988; Gleave et al., 1993). Eighty percent of all bladder TCC is superficial at presentation; the remaining 20% is muscle invasive and 50% of patients in this category die despite treatment (Simoneau and Jones, 1994). Of those patients initially presenting with superficial tumours, 50 to 70% have recurrences within two years. These recurrences are usually superficial, although 10 to 20% progress to the muscle invasive form (Parmer et al., 1989; Fradet, 1992; Harland, 1994).

The high frequency of recurrent TCCB and the increase in disease status in a proportion of patients means

that lifetime follow-up using cystoscopy and urinary cytology is essential. The standard procedure is an initial check cystoscopy three months after disease presentation; if this is clear cystoscopy should then be carried out every six months, for one to two years and then annually thereafter with a flexible cystoscope. At present the recurrence rate of TCCB means that annual lifetime cystoscopies should be carried out for all stabilised patients.

Cystoscopy involves insertion of a cystoscope into the bladder via the urethra to allow visualisation of the tumour using fibre optics. It confirms clinically and pathologically the presence of tumour within the bladder and allows a morphological description (Hossan and Striegal 1993). However it has the disadvantages of being an invasive, uncomfortable procedure. The frequent recurrences of TCCB mean that patients must undergo lifetime follow-up using cystoscopy; this results in the further disadvantage of a large expenditure by the health service.

Urine cytology is used for the detection of recurrent bladder TCC and although it offers the advantages of being a non-invasive, inexpensive, easily accessible procedure (Zein and Milad, 1991), it has a poor sensitivity, especially at lower stages and grades of disease. The result is false positive and negative findings with reported sensitivities ranging from 37.9% (Miyanaga eta al., 1997) to 64% (Martins et al., 1997).

Numerous studies have been carried out to find the ideal bladder cancer marker. However, none are adequately sensitive or specific enough to fulfil a diagnostic role at present. The most successful to date appears to be the Bard BTA, STAT and TRAK tests

with overall sensitivities of 55% (Bard promotional information), 72% (Leyh et al., 1997) and 88% (Bard promotional information) respectively.

Bladder cancer is a frequently recurring disease; patients require lifetime monitoring using cystoscopy and urinary cytology. Cystoscopy is an invasive technique and urinary cytology while non-invasive has a low sensitivity.

It is an aim of the present invention to replace these two procedures with a sensitive, non-invasive urinary test which would allow detection of first presentation and recurrent bladder cancer.

The invention relates to the presence of a 37KDa epidermal growth factor receptor (EGFR) fragment in the urine of patients with transitional cell carcinoma of the bladder (TCCB) and in the urine of some patients with prostate cancer.

This fragment had not previously been detected and its presence permits the development of a novel and inventive diagnostic test.

The 37KDa fragment can be observed in a western blot of proteins from a urine sample from a patient with TCCB.

According to the present invention there is provided a marker for bladder cancer, the marker comprising a 37KDa EGFR fragment which is detectable in urine.

The marker may also or alternatively be used as a marker for prostate cancer.

The invention provides a test for the presence of a

37KDa EGFR fragment in urine, the test comprising detecting the 37KDa EGFR fragment with an antibody. The test may comprise a western blot assay.

Alternatively the test may comprise an immunochromatographic assay, an ELISA test, latex agglutination or radioimmunoassay.

The invention further provides a method of diagnosing bladder cancer or prostate cancer or detecting recurrence of these, the method comprising the steps of reacting a urine sample from an individual to be tested with means to detect a 37KDa EGFR fragment and analysing results.

Herein the term "diagnosing" relates to first presentation diagnosis and detection of recurrence.

In one embodiment the means to detect the 37KDa EGFR fragment is an antibody.

Preferably the antibody is raised against a peptide corresponding to amino acid residues 1005 to 1016 of EGFR or binds to such a peptide or a peptide substantially similar thereto.

A substantially similar peptide is 60% homologous to the amino acid sequence along at least 50% of the length of the 37KDa peptide.

In a particular embodiment of the invention the antibody is Ab4 EGFR antibody available from Oncogene Science, Inc.

The invention further provides the use of antibody Ab4 EGFR in a test to detect the present of 34KDa EGFR

fragment in urine.

The invention also encompasses the use of specific antibodies raised to the 37KDa fragment of EGFR.

In one embodiment the test is in the form of a dip stick.

The test can be used in conjunction with other appropriate tests to diagnose TCCB, prostate cancer and urinary infection.

Experiment 1

A 37KDa EGFR fragment has been detected in urine from patients with bladder cancer. First morning urine samples were collected from 24 TCC patients, 6 patients who had bladder cancer previously but who were now disease free and 13 healthy volunteers. 10mls of urine from each was freeze dried and the powdered residue reconstituted in Laemmli lysis buffer. After heating at 110°C for 20 minutes, all samples were stored at -70°C until required for analysis. Samples were then probed with the Ab4 EGFR antibody (Oncogene Sciences) to the internal domain of the receptor by western blot analysis.

Disease Status	No	Presence of the 37KDA Fragment	Absence of the 37KDA Fragment
Healthy	13	1	12
TCC	24	21	3
Remission (disease free)	6	4	2

A 37KDa fragment was detected in 88% (21/24) of TCC patients, 66% (4/6) of disease free patients and 7% (1/13) of healthy volunteer urine samples. There was

an overall significant association between detection of the 37KDa fragment and presence of bladder cancer. Although four out of six patients who were though to be disease free tested positively, two had frank low grade tumours and two had bladder inflammation at the time the urine sample was taken. This 37KDa fragment therefore appears to be of diagnostic importance. It has a much higher sensitivity than urinary cytology and the Bard BTA and STAT tests, and it appears to be comparable to the Bard TRAK test.

Experiment 2

Disease Status	Number†	Presence of the 37KDA Fragment	Absence of the 37KDA Fragment	$(CHI)^2$
Healthy	25(13)	1(4%)	24(96%)	
Urinary Infection	16(12)	10(62.5%)	6(37.5%)	
Remission (disease free)	6(2)‡	0	6(100%)	46.17*
TCC	32(24)	28(87.5%)	4(12.5%)	
Prostate Cancer	10(0)	5(50%)	5(50%)	

Sensitivity levels for the detection of a 37KDa EGFR fragment in urine.

* denotes significant ($p<0.001$); †number in brackets is the number originally reported.

‡ This is somewhat different from Experiment 1 - the 6 so called remission patents were in fact all in remission when the notes were checked.

In fact: two were in remission, BUT two had

inflammation and two frank low grade tumour - and have been reassigned. Four more patients who are definitely in remission at the time of the test were added and there are now 6 confirmed remission patients with no marker.

Overall the second study has increased the number by a small amount and the data is holding up well. A group of prostate cancer patients has been added in since males often have undiagnosed prostate cancer. This could be a confounding factor as 50% are positive. However there is a blood test for prostate cancer so this would have to be carried out on positive patients along with a check for infection.

It is possible that the 37KDa protein could be used to distinguish between stage or grade in prostate cancer. The biology of prostate should be clarified and then collated with the patients tested. The test could be used as a general screen for health in the genitourinary area since it might pick up silent bladder and prostate tumours and infection - a positive test could lead to other tests to rule these possibilities out.

Comment on the table:

- shows 87.5% of TCC patients tested positive for the protein, whereas in contrast only 4% of the healthy controls expressed this protein in urine

- those patients in disease free (in remission), 100% tested negative

- the urinary infection group, 62.5% of the patients tested positive and 37.5% tested negative

- 50% of the prostate cancer patients test positive

- to date, the overall sensitivity of the 37KDa protein is 87% and the specificity is 96%.

- statistical analysis shows that detection of the 37KDa fragment is dependent on the presence of disease ($chi^2=46.17$ $p<0.001$).

Detection of the 37KDR EGFR fragment in urine

From the investigations carried out on the detection of the 37KDa EGFR fragment, it has been statistically established that the detection of the protein is dependent on disease presence. The fact that all remission patients analysed, tested negative for the 37KDa fragment is very encouraging. To date the overall sensitivity of the fragment protein is 87% and the specificity is 96%. Both these figures are superior to those of the BTA stat and the NMP22 tests which are commercially available. The sensitivities for the NMP22 and the BTA stat are 48% and 57% respectively, with specificites of 70% and 68% respectively (Weiner *et al*, 1998). However, the 37KDa EGFR fragment test is not 100% sensitive or specific. The test did not pick up 4 patients who had bladder tumours at the time of analysis. It may therefore be suggested that the 37KDa test could be used in tandem with both the NMP22 and the BTA stat test to reach 100% sensitivity and specificity. If 2 out of 3 of the tests gave positive results for a particular patient, it could be predicted that the patient had a bladder tumour. However, this hypothesis needs to be researched further, in order for this statement to be confirmed.

The test of the present invention may be used alone or together with any other suitable test.

Of the prostate patients analysed, 50% tested positive for the 37KDa fragment. The medical records of these patients will have to be researched further to confirm if they also had a undetected bladder tumour at the time of urine analysis. If it is found that these patients did not have bladder cancer, they could be ruled out by performing the prostate-specific antigen (PSA) test.

From the data obtained it was also found that 57% of urinary infection patients tested positive for the 37KDa fragment. This was to be expected, as EGFR over expression has been associated with inflammation and chronic irritation (Uhlman *et al.*, 1996). The urinary infection patients would have to be treated with a course of antibiotics before the 37KDa test could be carried out. The 37KDa fragment test has a number of clinical uses. Firstly, the test could be used to determine whether or not a patient requires cystoscopy. This would cut down on the number of cystoscopies presently carried out and would save the National Health Service considerable expense. The test would also be less traumatic for the patient than having cystoscopy, which is an uncomfortable, time consuming procedure. As males are becoming more aware of their own health, the test could also be used to screen males over 50 years, as this is the group most at risk from bladder cancer. It is hoped that a urinary dip-stick will allow quick detection of the presence of a bladder tumour.

The high frequency of recurrent TCC in the bladder and the progression to a more malignant phenotype in a

proportion of patients means that lifetime follow-up using cystoscopy and urinary cytology is essential. Cystoscopy is an invasive procedure and urinary cytology while non-invasive is relatively insensitive. At present the Bard BTA and STAT tests are the only commercially available detectors for bladder cancer. Their sensitivity means that at best they will only act in conjunction with cystoscopy. The Bard TRAK test while more sensitive has yet to be marketed and in fact the results from the present study indicate that the 37KDa EGFR fragment is at least comparable. Further work is required to investigate the significance of this fragment in the detection of first presentation and recurrent bladder TCC and to determine whether making it into a quantitive test will offer some insight into prognosis. Appropriate applications are detailed below.

The 37KDa EGFR fragment may be used as a detector for first presentation bladder and recurrent bladder TCC. Detection of the 37KDa EGFR fragment may be carried out by other methods of investigation as well as western blot analysis. These methods may include immunochromatography, ELISA, latex agglutination or radioimmunoassay. There is currently available a one-step immunochromatographic assay which qualitatively detects bladder tumour antigen in urine in five minutes. Detection of the 37KDa EGFR fragment may be detected by a similar method. Patient urine would be added to the small chamber where it mixes with a colloidal gold-conjugated antibody. If the 37KDa fragment is present, a 37KDa fragment conjugate complex would form. The reaction mixture would flow through the membrane which contains zones of immobilised capture antibodies. In the test zone, the 37KDa fragment conjugate complexes would be captured by a

second antigen-specific antibody, forming a visible line. If the 37KDa fragment is not present in the urine, no visible line would form.

EGF-Receptor (Ab-4) is available from Oncogene Science, Inc. as catalogue no. HCS16. There is no suggestion that the antibody could be used to diagnose the presence of the 37KDa EGFR fragment in urine or that the presence of this fragment is indicative of bladder or prostate cancer.

Other antibodies can be developed which are specific to the 37KDa fragment. This may increase sensitivity of the test.

A dip-stick test may be developed. This may require using methods such as latex agglutination, immunochromatogrphy, ELISA and radioimmunoassay.

Bladder cancer prognosis has been correlated with a number of factors, the single most important of which is depth of invasion of the bladder wall (Gospodarowicz, 1995); this is followed by grade of tumour (Heney et al., 1983). Other less important factors which influence patient outcome include tumour size (Gospodarowicz, 1995), age of patient at diagnosis (Fitzpatrick and Reda, 1986) and health status (Thrasher et al, 1994). None of these factors can predict prognosis in 100% of patients and so the 37KDa fragment may have some use prognostically. The EGFR fragment may be detected quantitatively using densitometry following western blot analysis and used to predict whether increased levels indicate a better or worse prognosis. Other quantitative methods may be developed to allow easier performance e.g. ELISA or radioimmunoassay techniques.

EGF and EGFR have been implicated in the pathogenesis of solid tumours such as those of the breast. This simple test developed for urine of patients with suspected TCCB might also be used to identify the diagnostic prognostic role of serum EGFR in other tumour types.